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Assessment of Opioid-Induced Immunomodulation in Experimental and Clinical Sepsis

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CONTEXT: Opioids remain a standard supportive therapy in patients admitted to the ICU with sepsis. However, as preclinical models indicate an association between opioid exposure and immunosuppression, the use of this class of drugs warrants investigation. The objective of this study was to investigate whether opioid exposure causes immunosuppression in patients with sepsis, and to use a murine sepsis model to determine the effects of opioid exposure on secondary infection.

HYPOTHESIS: We hypothesized opioid exposure would be associated with immunosuppression in patients with sepsis and secondary infection in a murine sepsis model.

METHODS AND MODELS: This was a two-phase preclinical and clinical study. The clinical phase included a subgroup of patients with sepsis from an existing randomized controlled trial while the preclinical phase used a murine model of sepsis with C57BL/6 mice. In the clinical phase, a post hoc analysis was performed in subjects receiving fentanyl versus no opioid receipt. In the preclinical phase, a murine cecal slurry-induced sepsis model followed by secondary infection was used. Mice were randomized to fentanyl versus no fentanyl concomitantly.

RESULTS: In clinical sepsis, a significant decrease in interleukin-23 (IL-23) level in patients with fentanyl exposure was observed and lower IL-23 was associated with mortality ($p < 0.001$). Other measured cytokines showed no significant differences. Concomitant fentanyl exposure during murine sepsis was associated with a significantly higher bacterial burden ($p < 0.001$) after secondary infection; however, immune cell counts and plasma cytokine levels were largely unaffected by fentanyl.

INTERPRETATION AND CONCLUSIONS: Minimal alterations in cytokines were seen with opioid exposure during clinical sepsis. In a preclinical model, opioid exposure during sepsis was associated with ineffective bacterial clearance upon secondary infection. Further studies are warranted to evaluate the immunomodulatory role of opioids and their implications, especially in the post-sepsis period.

KEY WORDS: analgesics; critical illness; fentanyl; immunomodulation; sepsis

Sepsis is a life-threatening condition caused by the body's exaggerated response to an infectious insult (1). If untreated, sepsis can progress to septic shock, which involves hemodynamic instability and is a leading cause of global mortality with up to 19 million cases yearly (1, 2). Despite mortality improvements over the past decade, outcomes for survivors are still considered suboptimal. Roughly 40% of survivors require rehospitalization within 30 days of discharge, most commonly for secondary infections (3).

Currently the limited first-line therapeutic options for septic shock include IV fluid resuscitation and/or vasopressors to restore hemodynamics,

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KEY POINTS

Question: Does concomitant fentanyl exposure cause immunosuppression in preclinical and clinical sepsis populations?

Findings: Concomitant fentanyl exposure during murine sepsis was associated with a significantly higher bacterial burden after secondary infection; however, immune cell counts and plasma cytokine levels were largely unaffected by fentanyl in both clinical and preclinical populations at the measured timepoints.

Meanings: Minimal alterations in cytokines were seen with opioid exposure during clinical sepsis at the measured timepoints.

anti-infectives for microbial control, and supportive care (3). Most commonly, opioids, such as fentanyl, morphine, and/or hydromorphone, are used for pain management. While opioids seek to provide analgesic action through acting on opioid receptors in the CNS, such receptors can also be found in both the immune and gastrointestinal systems (4). Thus, these drugs may have off-target effects on both innate and adaptive immune responses that are vastly understudied in the context of sepsis (4). Specifically, opioid exposure has been shown to inhibit lymphocyte, macrophage, and natural killer cell activity, alter toll like receptor signaling, suppress myeloid cell differentiation, and affect T cells resulting in a type 2 T helper (Th2) shift (4). The Th2 shift is a known consequence of late sepsis, with the potential to contribute to secondary infections in the ICU (5, 6). Unfortunately, no trials have evaluated whether short-term alterations correlate with long-term infectious outcomes, particularly with the most used opioid in the ICU, fentanyl (7).

Further limiting the advancement of this therapeutic question is the lack of translational animal models appropriate for investigating long-term outcomes after sepsis (8). Utilizing an innovative model of severe sepsis with long-term survival, we sought to investigate the effects of opioid exposure during sepsis on secondary infection post-sepsis (9, 10). This model is a critical tool for studies seeking to evaluate long-term outcomes, since traditional sepsis models are either too severe to result in long-term survivors or too weak to cause long-term complications. To date, no studies have evaluated the immunologic effects of opioid exposure on long-term outcomes in septic shock.

In this study, we surveyed the effect of opioid exposure on cytokine levels during clinical sepsis, and after secondary infection post-sepsis in a murine model. Our study suggests that opioid treatment during sepsis has minimal effects on acute or chronic plasma cytokine levels but does impair bacterial clearance in the post-sepsis period.

METHODS

Patient Population

A post hoc analysis of a previous randomized controlled clinical trial, Higher versus Lower Positive End-Expiratory Pressures in Patients with Acute Respiratory Distress Syndrome (ALVEOLI), was conducted (11). Biospecimens and patient data were requested from the National Heart, Lung, and Blood Institute Biologic Specimen and Data Repository Information Coordinating Center from the parent study. Specifically, ALVEOLI subjects with sepsis with at least 0.1 mL of plasma from day 0 (baseline day of ALVEOLI enrollment less than 48 hr from onset of acute lung injury) and day 3 (defined as the third study day after ALVEOLI enrollment) were included. We included only patients with a primary diagnosis of sepsis as determined by the original study investigators and excluded patients with an alternative primary diagnosis or those treated with any opioid other than fentanyl during the study period. Patients were divided into two groups: fentanyl use and no opioid exposure. Patients were included within the ALVEOLI trial if they were intubated and receiving mechanical ventilation with a decrease in the ratio of the partial pressure of arterial oxygen to the F_{IO_2} of 300 or less, a recent appearance of bilateral pulmonary infiltrates consistent with the presence of edema, and no clinical evidence of left atrial hypertension (defined by a pulmonary-capillary occlusion pressure of 18 mm Hg or less, if measured). Patients were excluded if 36 hours had elapsed since the eligibility criteria were met, younger than 13 years old or pregnant, and active in other trials involving acute lung injury within the preceding 30 days (11). Additional exclusion criteria can be found on the original study protocol. All human studies were approved by the University of Kentucky Institutional Review Board (Approval Date February

22, 2021, No. 55747, Impact of Opioid Exposure on Immune Function in Translational Sepsis Models) with waiver of informed consent. All procedures were in accordance with the Institutional Review Board's ethical standards on human experimentation and with the Helsinki Declaration of 1975.

Cytokine Assessment—Clinical

Cytokines were measured in plasma obtained from patients on day 0 and day 3 after study enrollment, as defined in the ALVEOLI protocol. The Bio-Plex Pro Human Th17 Cytokine Panel 15-Plex, which includes interferon gamma, interleukin (IL)-10, IL-17A, IL-17F, IL-1β, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IL-4, IL-6, tumor necrosis factor (TNF), and soluble CD40 ligand was used. Assays were run on the BioPlex 3D Suspension Array System (Bio-Rad Laboratories, Hercules, CA) per manufacturer's protocol. Any values below the limit of detection were imputed as the lower limit of detection. Limits of detection can be found in the **Supplementary Appendix** (http://links.lww.com/CCX/B131).

Animals and Husbandry

Male C57BL/6 mice were obtained from the National Institute on Aging and used at 12 months of age. Upon arrival, all mice were housed in pressurized intraventilated cages with free access to drinking water and chow (Teklad Global No. 2918) and maintained in an environment under controlled temperature (21–23°C), humidity (30–70%), and lighting (14 /10 hr, light/dark). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and performed in accord with the National Institutes of Health guidelines for ethical animal treatment.

Preclinical Sepsis Model

Sepsis was initiated by intraperitoneal injection of cecal slurry (CS, 500 mcL [100 mg]), prepared as previously described (9, 10). Antibiotics (imipenem; 1.5 mg/mouse, intraperitoneal) and fluid resuscitation (700 mcL 0.9% sodium chloride) were administered beginning 12 hours following CS injection and continued bid for 5 days. The dose of CS used would achieve LD100 if administered without subsequent therapeutic resuscitation. Mice were randomized to receive fentanyl (0.02 mg/kg) or vehicle (equal volume of saline) intraperitoneal bid for two weeks beginning at 12 hours post-CS injection. Changes in body weight and rectal body temperature (Digi-Sense, Kent Scientific, Torrington, CT) were assessed; mice not developing severe hypothermia (< 30°C) were excluded from the study. Two weeks later, a secondary infection was induced by injection of lower dose CS (250 mcL, 50 mg, LD50). Blood was aseptically collected by tail vein micropuncture and cultured on agar plates for assessment of bacteremia, as previously described (9, 10). A second murine experiment was performed in which the same model was used to evaluate immune response. Nonsepsis control mice receiving vehicle injection (10% glycerol, intraperitoneal) and fluid resuscitation (no antibiotics) were included. Six hours after secondary infection, mice were deeply anesthetized by isoflurane inhalation, laparotomy performed, and blood collected from the inferior vena cava by syringe needle with 10% volume of 0.5 M EDTA. One-hundred mcL whole blood was reserved for complete blood count using IDEXX ProCyte Dx Hematology Analyzer. The remaining blood was immediately centrifuged (2,500 × g, 4°C, 15 min) to obtain plasma, which was stored at –80°C.

Cytokine Assessment-Mouse

Plasma cytokine concentrations were assessed using Mesoscale Discovery mouse V-Plex kits (K15048 and K15245) following the manufacturer's protocol. Limits of detection can be found in the **Supplementary Appendix** (http://links.lww.com/CCX/B131). In the initial survival study, 6-hour plasma IL-6 levels were assessed by nonlethal blood sampling (10 mcL) and enzyme-linked immunosorbent assay (Invitrogen, Waltham, MA, BMS603-2) per the manufacturer's protocol.

Statistical Analysis

For baseline criteria, p values for continuous variables were calculated using independent-sample t tests. p values for gender, diabetes, vasopressors, and ethanol use were calculated using chi-square tests. Due to low cell counts in the clinical samples, p values for the remaining categorical variables were calculated using Fisher exact tests. Due to the presence of large outliers, analysis of differences in cytokine level (day 3–day 0)

was implemented using robust regression with Huber weights. Given the established link between age and immune response, age was included in all models as a potential confounder. Within the murine studies, independent-sample *t* tests with a Bonferroni correction were performed to examine differences in average bacterial load between CS versus CS + fentanyl groups at each time point. Analyses for both complete blood count and cytokine values were assessed via Wilcoxon rank-sum tests. For each set of analyses, *p* values are reported unadjusted as well as adjusted using a Benjamini-Hochberg correction for multiple testing.

RESULTS

Clinical Phase

Within the clinical phase of this study, 101 total patients met inclusion criteria. Of these, 29 (28.7%) did not receive any opioids, and 72 (71.3%) received fentanyl. The groups were similar in major baseline characteristics except for population age and alcohol use (**Table 1**; and **eTable 1**, http://links.lww.com/CCX/B131). Levels of 15 cytokines were measured in the plasma from these patients at day 0 and day 3 (**Fig. 1**). After correction to control the false discovery rate at 0.05, the change in IL-23 between 0 and day 3 was the only notable different cytokine between patients receiving fentanyl versus those without opioid treatment, with IL-23 levels ranging lower in fentanyl patients; however, both had a median of 0.0 pg/mL (**Table 2**; and **eTable 2**, http://links.lww.com/CCX/B131) Clinical outcomes between groups were not found except for a prolonged ICU stay in those patients receiving fentanyl (**Table 3**). However, among those who received fentanyl, there was a significant association between change in cytokine level and survival for all 15 cytokines except TNF; the adjusted *p* value for TNF was close to the predetermined threshold for statistical significance ($p = 0.061$) (**eTable 3**, http://links.lww.com/CCX/B131). Among those who received fentanyl, there was not a significant association between change in cytokine level and ICU

TABLE 1.
Clinical Patient Characteristics

Characteristic	No Opioids (*n* = 29)	Fentanyl (*n* = 72)	*p*
Age (yr), mean (SD)	58.7 (17.1)	50.1 (16.6)	0.024
Female gender, *n* (%)	14 (48.3)	31 (43.1)	0.798
White, *n* (%)	24 (82.8)	50 (69.4)	0.478
Chronic dialysis, *n* (%)	1 (3.4)	4 (5.7)	> 0.999
AIDS, *n* (%)	0 (0.0)	5 (7.1)	0.318
Leukemia, *n* (%)	2 (6.9)	1 (1.4)	0.204
Non-Hodgkin's lymphoma, *n* (%)	0 (0.0)	1 (1.4)	> 0.999
Solid tumor with metastasis, *n* (%)	0 (0.0)	1 (1.4)	> 0.999
Immune suppression, *n* (%)	5 (17.2)	10 (14.3)	0.761
Hepatic failure, *n* (%)	0 (0.0)	1 (1.4)	> 0.999
Cirrhosis, *n* (%)	1 (3.4)	2 (2.9)	> 0.999
Diabetes mellitus, *n* (%)	5 (17.2)	13 (18.6)	> 0.999
Vasopressors last 24 hr, *n* (%)	12 (41.4)	34 (47.2)	0.755
Ethanol use, *n* (%)	1 (3.8)	17 (25.8)	0.036

The following variables had two missing observations each: AIDS, cirrhosis, diabetes mellitus, chronic dialysis, ethanol use, hepatic failure, immune suppression, leukemia, non-Hodgkin's lymphoma, and solid tumor.

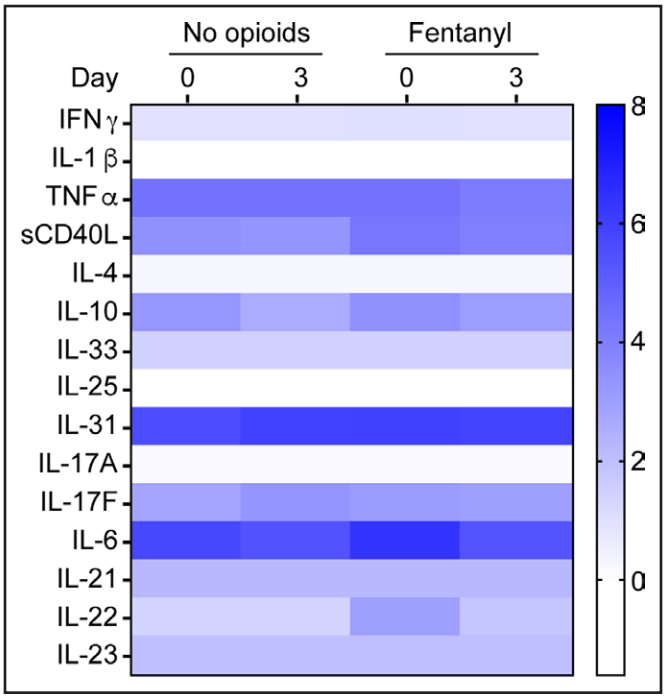

Figure 1. Cytokine profile within human sepsis is minimally affected by fentanyl exposure during the first 72 hr of hospitalization. Heatmap depicting global changes in cytokines in clinical sepsis. Values reflect the log of the median cytokine level. IFNγ = interferon gamma, IL = interleukin, sCD40L = soluble CD40 ligand, TNFα = tumor necrosis factor alpha.

TABLE 2.
Clinical Cytokine Analysis

Cytokine	No Opioids ($n = 29$)	Fentanyl ($n = 72$)	p
Interferon gamma, difference	0.0 (0.0–9.9)	0.0 (–8.9 to 0.0)	0.14
IL-10, difference	0.0 (–9.1 to 4.4)	–5.2 (–33.4 to 0.6)	0.18
L-17A, difference	0.0 (–0.9 to 0.0)	0.0 (–4.6 to 0.0)	0.99
IL-17F, difference	0.0 (–5.1 to 28.5)	0.0 (–20.7 to 13.9)	0.36
IL-1β, difference	0.0 (0.0–0.9)	0.0 (–1.1 to 0.2)	0.11
IL-21, difference	0.0 (0.0–10.8)	0.0 (–19.4 to 0.7)	0.19
IL-22, difference	0.0 (–3.3 to 34.7)	0.0 (–57.2 to 0.0)	0.18
IL-23, difference	0.0 (0.0–54.9)	0.0 (–23.5 to 0.0)	0.0468
IL-25, difference	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.51
IL-31, difference	40.2 (0.0–508.4)	–32.7 (–289.4 to 154.1)	0.14
IL-33, difference	0.0 (0.0–52.8)	0.0 (–12.8 to 0.0)	0.14
IL-4, difference	0.0 (0.0–2.8)	0.0 (–1.6 to 0.0)	0.18
IL-6, difference	–86.5 (–450.8 to 16.3)	–295.3 (–1,417.9 to –42.0)	0.18
Tumor necrosis factor alpha, difference	–18.9 (–41.3 to –0.4)	–9.7 (–55.8 to 10.9)	0.53
Soluble CD40 ligand, difference	0.0 (–9.8 to 123.5)	–0.4 (–78.5 to 0.7)	0.11

IL = interleukin.

All values in Table 2 are displayed as median (1Q–3Q). All units are in pg/mL. Differences in the table represent changes in cytokine levels (day 3–day 0). *p* values correspond to the association between groups (fentanyl vs no opioids) and change in cytokine level from the robust regression model, after controlling for age and adjusting for multiple testing.

TABLE 3.
Clinical Outcomes

Characteristic	Control ($n = 29$)	Fentanyl ($n = 72$)	p
Survival to hospital discharge	28 (96.5)	63 (87.5)	0.168
ICU readmission	3 (10.3)	8 (11.1)	0.562
ICU duration, d	7 (9)	9 (14)	0.020
Hospital duration, d	15 (17)	18 (17)	0.109

Continuous variables in Table 3 are displayed as median (1Q–3Q) with yes/no variables reported as rates and percentages.

duration for any of the 15 cytokines (**eTable 4**, http://links.lww.com/CCX/B131).

Murine Phase

Sepsis was induced in mice (n = 18) by intraperitoneal administration of CS, with half given fentanyl according to the study timeline (**Fig. 2*A***). The characteristic hypothermic response observed in murine sepsis models was observed in both groups immediately after CS injection, with no significant difference between CS + vehicle versus CS + fentanyl groups (p = 0.128) (**Fig. S1*B***, http://links.lww.com/CCX/B131). Following initial sepsis, body weight similarly decreased in both groups ($p < 0.001$), with no significant difference between the groups (p = 0.367) (**Fig. S1*C***, http://links.lww.com/CCX/B131). Mortality was 11% in the vehicle group (one of nine mice died), while no mice died in the fentanyl group (p = 0.3173) (**Fig. S1*A***, http://links.lww.com/CCX/B131). Upon secondary infection, a similar, but dampened, hypothermic response was observed with no significant difference between the groups (p = 0.084) (**Fig. S1*D***, http://links.lww.com/CCX/B131). To assess severity of initial sepsis as well as the secondary infection, IL-6 was measured 6 hours after CS injection (12). During the initial response to sepsis (prior to fentanyl exposure), IL-6 levels were similar between vehicle and fentanyl groups (113.8 ± 57.0 vs 106.7 ± 39.3 ng/mL; p = 0.777). Following challenge with secondary infection (includes 2 wk of fentanyl exposure), vehicle-treated mice exhibited IL-6 levels

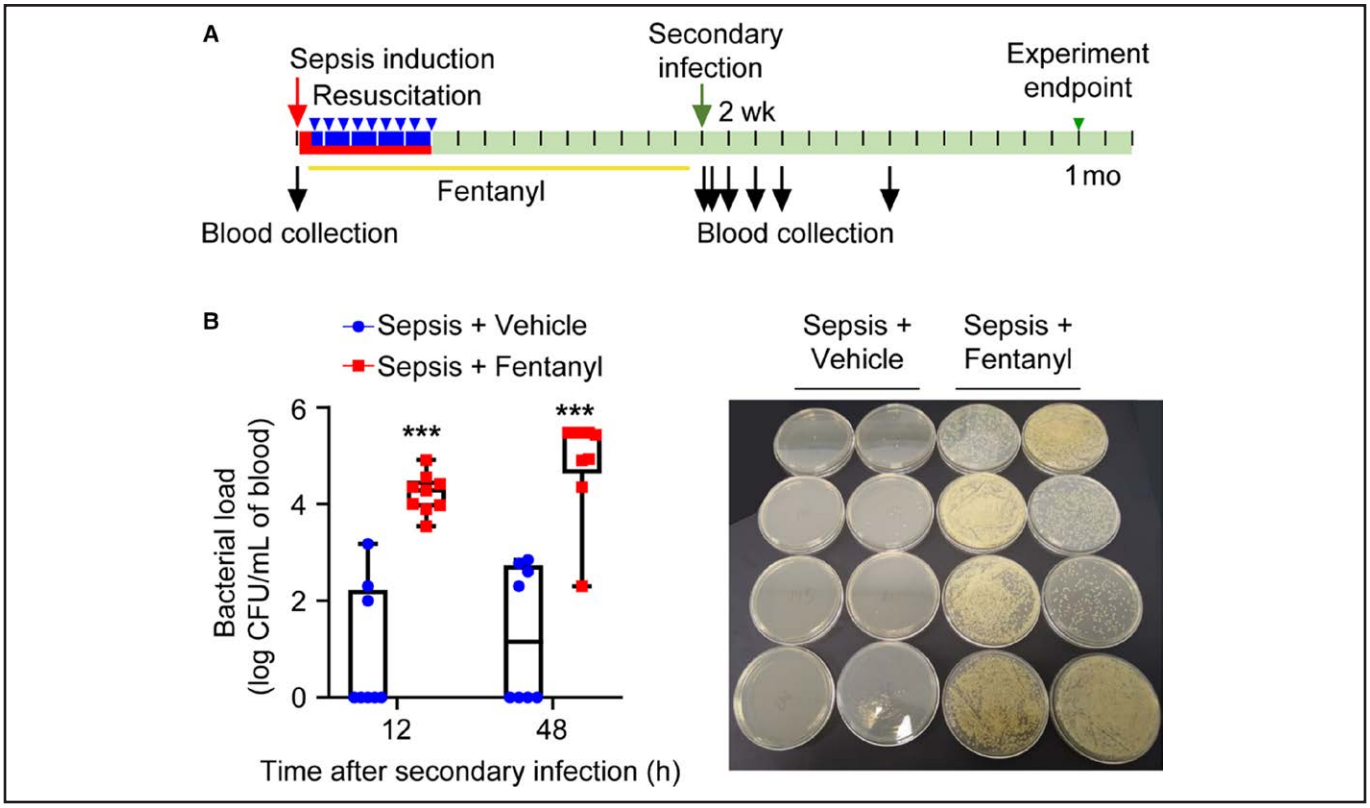

Figure 2. Bacterial load is greater in fentanyl-treated mice following a secondary infection. Mice were given sepsis by intraperitoneal injection of cecal slurry (CS) and randomized to fentanyl and vehicle groups. Two wk later a secondary infection was induced. **A**, Schematic of experimental timeline. **B**, Blood bacterial load 12 and 48 hr after secondary infection with depiction of bacterial colonies on agar plates. Data are expressed as individual values in *box plots* with a *bar* indicating the mean. ***$p < 0.001$. CFU = colony forming units.

similar to the initial challenge (108.8 ± 66.1 ng/mL; $p = 0.861$), while IL-6 levels in fentanyl-treated mice were significantly reduced when compared with vehicle control (41.1 ± 42.1 ng/mL; $p = 0.041$) (**Fig. S1*E***, http://links.lww.com/CCX/B131). Furthermore, fentanyl-treated mice had a significantly higher bacterial burden compared with vehicle-treated mice at 12 hours (24,078 vs 225 Colony Forming Units/mL, respectively) and 48 hours (126,500 vs 238 Colony Forming Units/mL) following secondary infection ($p < 0.001$) (**Fig. 2*B***).

To further evaluate the effect of prior fentanyl exposure on secondary infection, a similar experiment was performed in another set of mice ($n = 18$, $n = 4$ each for nonsepsis control groups, $n = 5$ each for sepsis + secondary challenge groups), and mice were euthanized 6 hours after the secondary challenge (**Fig. 3*A***, experimental timeline). Complete blood counts were assessed, which showed increased monocytes, neutrophils, and platelets in mice with the secondary infection but no significant difference in cell counts between groups receiving fentanyl versus vehicle (**eTable 5**, http://links.lww.com/CCX/B131). A panel of 28 cytokines were measured by multiplex analyses; most of the measured cytokines were elevated 6 hours after the secondary infection in both fentanyl-treated and vehicle groups compared with non-infected control mice (**Fig. 3*B***; and **eTable 6**, http://links.lww.com/CCX/B131). While there was a downward trend in the level of multiple cytokines in the fentanyl-treated group, only interferon gamma-induced protein 10 (IP-10), C-X-C motif chemokine ligand 10, macrophage inflammatory protein-1 alpha (MIP-1α), C-C motif chemokine ligand, and IL-1β were statistically significant (unadjusted p value). After adjusting for multiple testing, no significant differences were detected.

DISCUSSION

Cytokine analyses have been frequently used in sepsis populations to evaluate immune response, both in clinical and preclinical models. In this multiphase study, we: 1) assessed whether immunomodulation occurs secondary to opioid exposure during

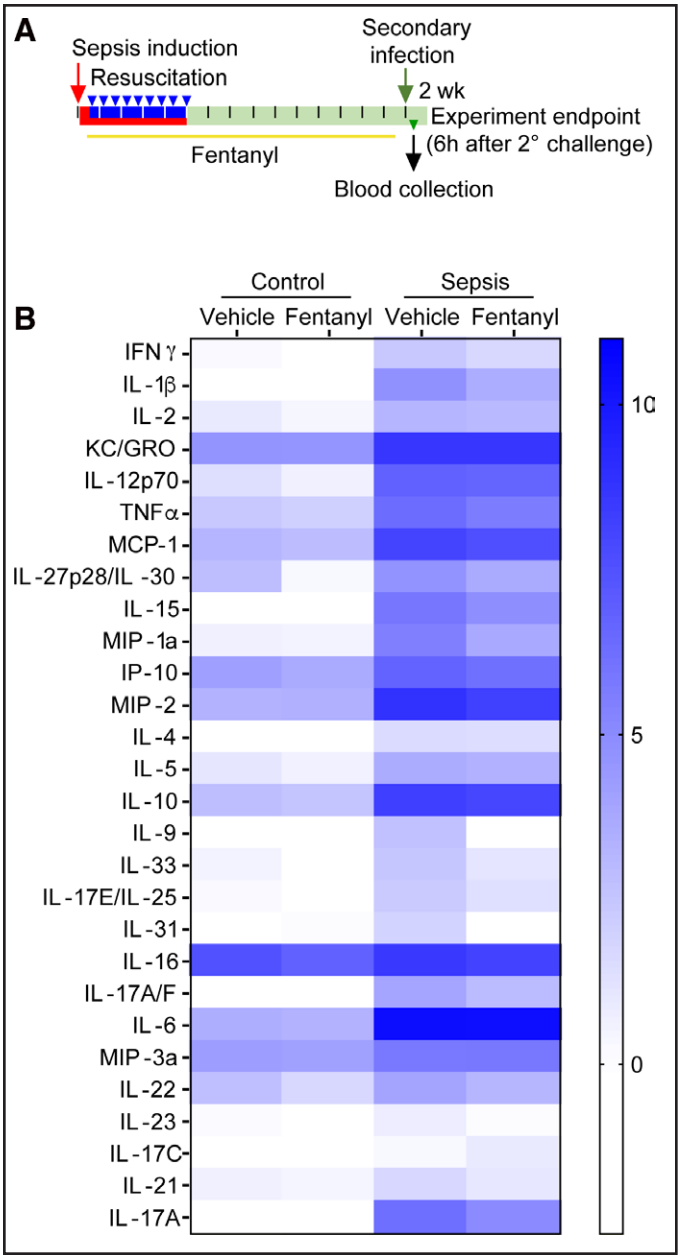

Figure 3. Cytokine profile 6 hr after secondary infection is minimally affected by fentanyl exposure during sepsis. Mice were given sepsis by intraperitoneal injection of cecal slurry (CS) and randomized to fentanyl and vehicle groups. Two wk later a secondary infection was induced. **A**, Schematic of experimental timeline. **B**, Heatmap depicting global cytokine changes 6 hr after secondary infection. Values reflect the log of the median cytokine level. IFNγ = interferon gamma, IL = interleukin, IP-10 = interferon gamma-induced protein 10, KC/GRO = Keratinocyte chemoattractant/human growth-regulated oncogene, MCP-1 = monocyte chemoattractant protein-1, MIP = macrophage inflammatory protein, TNFα = tumor necrosis factor alpha.

clinical sepsis via evaluation of cytokine profiles and 2) assessed whether opioid exposure during sepsis affects the immune response to a secondary infection using a preclinical model. With the exception of ICU length of stay, clinical outcomes were not significantly different and cytokine levels showed minimal alterations in patients with or without opioid exposure, precluding our ability to determine whether immunomodulation occurred in this patient population. In our animal model, a significant reduction in bacterial clearance after secondary infection was observed in those with fentanyl exposure during sepsis suggesting immunomodulation; however, cytokine levels were minimally affected.

Within this study, 3 days of fentanyl exposure did not significantly impact cytokine levels in patients compared with those without opioid treatment, with one exception. IL-23 was significantly reduced by fentanyl treatment and the decrease associated with mortality. This result aligns with prior work demonstrating that morphine causes dysfunction in IL-23 producing cells (13). IL-23 is important for the induction of neutrophil-mediated protective immune responses against pathogens, thus lower levels would impact bacterial clearance leading to more severe infection (13–15) While other cytokines showed no significant difference between the groups, a decrease or no change in cytokine level from day 0 to day 3 was associated with decreased survival in fentanyl-treated patients. While cytokine concentrations may be a useful assessment of systemic inflammation, they are inherently only a short, singular glimpse, therefore unable to show true functional state of individual lymphocyte and myeloid populations over time. In one previous study, high IL-1β levels were associated with increased early mortality, while our study demonstrated that IL-1β was more often decreased in those who died (16). Our study measured the change in IL-1β from enrollment to day 3, while Bozza et al (16) measured cytokines only on day 1, which could account for the different results. Within our clinical cohort, fentanyl was associated with a non-statistically significant lower rate of survival, and depression in cytokine profiles was specifically associated with increased rate of death in patients receiving fentanyl, which aligns with limited available evidence. One retrospective case-control study of critically ill patients demonstrated increased infectious complications in patients receiving the highest amount of opioid exposure; however, this study was limited by its patient population and high statistical fragility (odds ratio, 1.24; CI, 1.0–1.54) (17). Based on the trend

toward worsening survival with opioid exposure in our clinical study, it remains plausible that immunosuppression does occur within the sepsis population secondary to exposure of clinically relevant opioids. Previous studies analyzing cytokines within the sepsis population were limited by their lack of correction for multiple testing or limited cytokine analyses and therefore lack of consideration for interactions within statistical methods (18). Future studies surrounding immunomodulation as a treatment or as a predictor of outcome within sepsis may need to consider measurements at multiple timepoints over the disease course along with functional cell assays. Additionally, as the growing body of data suggests the importance of genomic signatures in sepsis and the potential genomic alteration of immune response in sepsis care, a multiomic approach may be best to fully understand this phenomenon (19–21).

Previous preclinical opioid studies have demonstrated numerous potential impacts of opioid exposure, including natural killer cell suppression, depression of antibody production, suppression of phagocytosis, apoptosis induction, inhibition of cell growth, and depression of the T cell mediated adaptive immune response (4). Using a preclinical CS-injection model of sepsis, we evaluated the effect of fentanyl exposure on secondary infection in the post-sepsis period. We chose the CS model, which has been validated in our laboratory, for a variety of reasons discussed at length elsewhere (9, 10). The primary benefit of CS as opposed to cecal ligation and puncture for this specific study is avoidance of the long-term effects of cecal ligation. As our goal was to study secondary infection in the post-sepsis period, we did not want the lasting effects of a ligated cecum to influence the outcome. This study is unique in that it is the first to look at the post-sepsis effects of fentanyl use in a murine model. The most significant finding was an increase in bacterial burden in the fentanyl-treated group, indicative of reduced bacterial clearance. However, we did not find significant differences in monocyte or neutrophil numbers, or major cytokines associated with bacterial containment as one would expect, suggesting a defect in immune cell function as opposed to number. Monocytes and neutrophils were increased by sepsis with secondary infection (regardless of fentanyl treatment) and many of the cytokines produced predominantly by these cells are those that were upregulated in sepsis compared with nonsepsis control. IL-1β, MIP-1α, and IP-10 are the only factors that were lower in the fentanyl compared with vehicle group (unadjusted *p* value). Lower levels of these cytokines could indicate reduced functionality of these cells and account for the decreased bacterial clearance in the fentanyl group. IL-1β has been shown to be protective against several bacterial infections by activating responses that lead to rapid neutrophil recruitment (22). MIP-1α and IP-10, which are both inducible by IL-1β likely play roles in this axis as they have also been implicated in promoting immune cell recruitment and bacterial clearance (23, 24).

Although both clinical and preclinical phases were included in this study, the design of each are not congruent; thus, we are unable to make direct comparisons. In the human phase, we aimed to study the effects of fentanyl exposure on immunosuppression during hospitalization for sepsis using available data and samples from an existing clinical trial. This study design had a 3-day window for plasma collection from study enrollment to day 3 after treatment. As this was an existing clinical trial, we were unable to include additional measurements. In the preclinical phase, we aimed to study the effects of fentanyl exposure during sepsis on post-sepsis immunosuppression and susceptibility to secondary infection. This study design allowed us to observe the increase in bacteremia in the fentanyl-treated group, suggesting that fentanyl exposure during a hospitalization for sepsis would increase risk of secondary infection. However, we were unable to determine whether fentanyl exposure increased risk for secondary infection in the clinical study as these data was not collected as part of the existing trial. A prospective study following patients after discharge would be necessary to determine whether fentanyl exposure during hospitalization increases risk of later secondary infection as was seen in our murine model. While we did not observe many significant differences in cytokines with fentanyl exposure in either the clinical or murine phases, these are not comparable as they were measured at different points in the study timeline (during sepsis vs during post-sepsis secondary infection).

There are many strengths to this study including the utilization of both preclinical and clinical populations, the multivariable methods used for cytokine analysis, selection for a highly clinically relevant opioid, and correction for multiple testing. However, there are important limitations to consider. Within both the

preclinical and clinical cohorts, sample sizes were limited. We opted not to perform a sample size analysis prior to this study given the nature of a post hoc analysis; however, the study could have benefited from additional patients if they were available. After correction of *p* values for multiple testing, we were underpowered to detect statistically significant differences in several of our study outcomes. Our cytokine analyses were limited to a single timepoint in the preclinical phase, and two early timepoints in the clinical phase, which did not allow us to draw conclusions about changes in cytokine levels at later times, which may have had a greater impact on immunomodulation. Another limitation, specific to the clinical population, is the lack of dose information regarding opioid exposure and the difficulty in assessing secondary infection. In reality, a number of limitations exist surrounding the identification of an infection in the clinical population, with almost half of all patients without a positive culture during sepsis. The ambiguity with this definition, in addition to a lack of standardized time points to define secondary infection, limit evaluation of this outcome (25–27). Additionally, while we adjusted our clinical cytokine analysis for age and multiple testing, additional potentially significant confounders could not be included due to the limited sample size.

CONCLUSIONS

In sepsis populations, fentanyl exposure may negatively alter immune function through a complex and unclear mechanism, increasing the risk of secondary infection. Our murine data show reduced bacterial clearance after a secondary infection, despite equivalent immune cell counts, suggesting that fentanyl exposure during sepsis impacts immune cell function post-sepsis. Our clinical population did not demonstrate differences in clinical outcomes or cytokine profiles with opioid exposure during acute sepsis. Future prospective analyses that evaluate the long-term impact of opioid treatment on immunosuppression in patients are needed to mitigate potential harm and to examine cellular alterations that may result from fentanyl exposure.

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All authors made substantial contributions to the conception or design of the work or the acquisition, analysis, or interpretation of data for the work. All authors all performed aspects of the preclinical experiments. Drs. Bissell, Sturgill, and Bruno performed the clinical experiment. Dr. Bissell drafted the article, and Drs. Sturgill and Starr revised it critically for important intellectual content. All authors provided final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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